REMARKS

Claims 16, 18-20, 22-30 and 32-36 are pending and under examination in the subject application. By this Amendment, applicants have canceled claims 16, 18-20, 22-27, 30 and 36, and amended claim 28. Applicants has herein amended claim 28 to more particularly point out what they consider to be the invention and applicants maintain that this Amendment raises no issue of new matter. Accordingly, upon entry of this Amendment, claims 28, 29 and 32-35 will be pending and under examination.

Rejection Under 35 U.S.C. §112, First Paragraph

Claims 16, 18-20, 22-30 and 32-36 were rejected under 35 U.S.C. §112, first paragraph, as allegedly not enabled for a method of reducing ischemic damage to a tissue being transplanted into a subject comprising contacting the tissue with any nucleic acid that inhibits the expression of Egr-1.

In response, and without conceding the correctness of the Examiner's rejection, applicants note that claims 16, 18-20, 22-27, 30 and 36 have been canceled. Accordingly, the Examiner's rejection of these claims is now moot.

Applicants respectfully traverse the rejection of claims 28, 29 and 32-35. Applicants direct the Examiner's attention to amended claim 28, which provides a "method for reducing ischemic damage to tissue being transplanted into a subject, which comprises contacting the cells of the tissue *ex vivo* with a nucleic acid comprising the polynucleotide sequence 5'-CTTGGCCGCTGCCAT-3' (SEQ. ID. NO: 1)

prior to the tissue's transplantation into the subject, wherein the nucleic acid inhibits Early Growth Response Factor-1 (Egr-1) expression in the cells of the tissue." Applicants note that the Examiner has conceded that the specification does enable "a method for reducing ischemic damage to lung tissue being transplanted into a subject comprising contacting the tissue with SEQ. ID. NO: 1 *ex vivo*." Applicants maintain that the use of a nucleic acid comprising SEQ. ID. NO: 1 to reduce ischemic damage in other types of tissue is also similarly enabled. In support of their position, applicants direct the Examiner's attention to Okada et al. (Transcriptional Control of Cardiac Allograft Vasculopathy by Early Growth Response Gene-1 (Egr-1), Circ. Res. 91: 135-142 (2002)), attached hereto as **Exhibit 1**.

In relevant part, Okada et al. found that ex vivo treatment of a *heart* with Egr-1 antisense at the time of cardiac harvest inhibits the expression of Egr-1 in cardiac allografts after transplantation. Additionally, Okada et al. found that cardiac allografts treated with Agr-1 antisense also resulted in substantially less neointimal formation. Applicants maintain that the findings in Okada et al. further support their position that one skilled in the art would have been able to practice the claimed invention at the time of filing.

In view of the above remarks, applicants maintain that claim 28, and dependent claims 28, 29 and 32-35 satisfy the requirements of 35 U.C.S. §112, first paragraph.

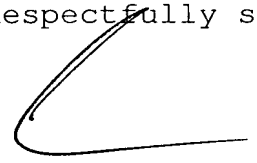
Summary

In view of the remarks made herein, applicants maintain that the claims pending in this application are in condition for allowance. Accordingly, allowance is respectfully requested.

If a telephone conference would be of assistance in advancing prosecution of the subject application, applicants' undersigned attorneys invite the Examiner to telephone them at the number provided below.

No fee, other than the enclosed $450.00 sum, is required. However, if any additional fee is required, authorization is hereby given to charge the amount of such fee to Deposit Account No. 03-3125.

Respectfully submitted,



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Transcriptional Control of Cardiac Allograft Vasculopathy by Early Growth Response Gene-1 (Egr-1)

Morihito Okada, Catherine Y. Wang, Daniel W. Hwang, Taichi Sakaguchi, Kim E. Olson, Yasushi Yoshikawa, Kanji Minamoto, Sean P. Mazer, Shi-Fang Yan, David J. Pinsky

Abstract—Expression of the zinc finger transcription factor early growth response gene-1 (Egr-1) is triggered rapidly after mechanical vascular injury or after a precipitous drop in ambient oxygen, whereupon it induces the expression of diverse gene families to elicit a pathological response. Initially characterized as an early response transcriptional activator, the role of Egr-1 in more chronic forms of vascular injury remains to be defined. Studies were designed to examine whether Egr-1 induction may serve as a causal link between early preservation injury and delayed vascular consequences, such as coronary allograft vasculopathy (CAV). The preservation and transplantation of heterotopic murine cardiac allografts strongly induce Egr-1 expression, leading to increased expression of its downstream target genes, such as intercellular adhesion molecule-1, vascular cell adhesion molecule-1, and platelet-derived growth factor A chain. Expression of these Egr-1–inducible gene targets is virtually obliterated in homozygous Egr-1–null donor allografts, which also exhibit attenuated parenchymal rejection and reduced CAV as long as 60 days. Congruous data are observed by treating donor hearts with a phosphorothioate antisense oligodeoxyribonucleotide directed against Egr-1 before organ harvest, which blocks subsequent expression of Egr-1 mRNA and protein and suppresses the late development of CAV. These data indicate that Egr-1 induction represents a central effector mechanism in the development of chronic rejection characterized by CAV. Blocking the expression of this proximal transcription factor solely at the time of organ harvest elicits beneficial delayed consequences for the cardiac allograft. **(*Circ Res.* 2002;91:135-142.)**

Key Words: early growth response-1 ■ antisense oligodeoxyribonucleotides ■ cardiac allografts ■ transcription factors ■ knockout mice

Cardiac transplantation represents a standard treatment for patients with end-stage heart failure, yet it remains an imperfect therapy. Coronary allograft vasculopathy (CAV), which develops in over half of all transplant recipients as little as 5 years after their procedures, poses the most significant limitation for their long-term survival.[1] CAV is characterized by diffuse intimal thickening composed of proliferative smooth muscle cells, matrix, and other cellular elements.[2] Although a number of immune and nonimmune mechanisms have been implicated in CAV pathogenesis, there remains considerable controversy as to the mechanistic basis for the development of CAV. The gross pathological hallmark of CAV, diffuse concentric narrowing of the lumen in allograft vessels, suggests that CAV development is driven by an immune-mediated process, but there are substantial data to suggest that nonimmune factors, such as ischemic injury, can accelerate or exacerbate its development.[3,4] Despite the presumed immunological basis, potent immunosuppressive regimens have had little impact on preventing CAV development or arresting its progression once identified.

In an attempt to define a potential unifying mechanism that may explain the contribution of diverse antigen-independent factors contributing to CAV pathogenesis, such as oxidant stress,[5] activation of coagulation,[6] and upregulation of adhesion receptors in allograft vessels,[7] we considered the role of a ubiquitous transcriptional activator responsible for the ischemia-driven activation of multiple gene cascades. Early growth response-1 (Egr-1), the product of an immediate-early gene and a prototypical member of the zinc finger family of transcriptional regulators, plays a pivotal role in the coordinated transcription of multiple inflammatory and coagulant genes, including ones that have been implicated in the pathogenesis of atherosclerosis and restenosis after vascular injury. These include interleukin-1β, transforming growth factor-β, intercellular adhesion molecule-1 (ICAM-1), tissue factor, plasminogen activator inhibitor-1, platelet-derived growth factor (PDGF)-A, and PDGF-B.[8,9] Egr-1 has also been localized to endothelial cells and smooth muscle cells in human atherosclerotic plaques,[10] and its expression is increased after mechanical vascular injury of the aorta[9] or carotid artery.[11] Furthermore, in a recent study of heterotopic cardiac allografts placed in Japanese (*Macaca fuscata*) monkeys, Egr-1 expression was detected in rejecting arteries at time points preceding the development of prototypical mor-

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phological vascular changes of CAV, raising the possibility of a causal link.[12]

To elucidate a potential mechanistic link between Egr-1 induction and CAV development, two complementary strategies were used in a heterotopic mouse model of cardiac transplantation. In the first, an antisense approach was used because of the recognized specificity and potency of the technique, particularly as applied to ex vivo preservation of an organ.[13–15] In the second approach, mice null for the Egr-1 gene, which exhibit reduced pulmonary vascular inflammatory and coagulant responses to oxygen deprivation or ischemia,[8] served as cardiac allograft donors. Theoretically, a multipronged approach to inhibit many inflammatory and coagulant cascades, by targeting a common transcription factor such as Egr-1 rather than the often-redundant mediators themselves, might be preferable to prevent parenchymal rejection and inhibit formation of CAV. These studies elucidate the pathophysiological role of Egr-1 in CAV pathogenesis.

Materials and Methods

Animals

Male mice aged between 8 and 12 weeks were used for these experiments. For allograft or isograft experiments, C57BL/6J (H-2^b) or B10A (H-2^a) mice were used as donors, and B10A mice were used throughout as recipients. For the antisense and scrambled-sequence oligodeoxyribonucleotide (ODN) control experiments, B10A mice were used throughout as donors, and C57BL/6J mice were used as recipients. For experiments in which Egr-1–null (Egr-$1^{-/-}$) donor mice were used, littermate control wild-type (Egr-$1^{+/+}$) donor mice were used; the genotypic identity of each mouse was confirmed by Southern blotting. The background of these mice is 129×C57BL/6J,[8] all of which have an H-2^b genotype. For these experiments, B10A mice were used throughout as recipients.

Antisense and Scrambled-Sequence ODNs

Antisense and scrambled-sequence phosphorothioate ODNs of 20-bp length were commercially synthesized and purified by using high-performance liquid chromatography (Operon Technologies). The Egr-1 antisense ODN was composed of the following sequence: 5′-GCGGGGTGCAGGGGCACACT-3′.[16] For control experiments, a scrambled sequence was used: 5′-TGCGAGGCCGGAGGGA-GCCT-3′. Antisense Egr-1 or scrambled-sequence ODNs were transfected into grafts during the harvest and preservation period by using a cationic liposomal carrier that has previously been shown to be an effective delivery vehicle in the lungs.[13,15]

Cardiac Transplant Experiments

Experiments were performed according to a protocol approved by the Institutional Animal Care and Use Committee at Columbia University. Transient immunosuppression was performed by preoperative administration of anti–murine CD4 (clone GK1.5) and anti-CD8 (clone 2.43) from hybridoma supernatants (American Type Culture Collection). These antibodies were injected intraperitoneally into recipients at days 6, 3, and 1 before the transplantation to permit graft survival of a sufficient duration so as to observe the formation of CAV. The ODN delivery solution was prepared by adding 100 μg of cationic liposomal carrier to 0.5 mL of lactated Ringer's solution (LR) at room temperature. Separately, 100 μg of either the scrambled-sequence or the antisense ODN construct was added to 0.5 mL of LR.[13,15] The delivery solution and the ODN construct solutions were mixed, incubated for 30 minutes at room temperature, diluted with 1.0 mL of LR, and then chilled to 4°C.

The transplantation procedure was performed as described in a previous study.[3,17] The heart was rapidly harvested after arrest with hypothermic potassium cardioplegia solution given via the inferior vena cava (1 mL, 20 mEq/L), the coronary arteries were flushed (0.5 mL of preservation solution), and the harvested heart was placed into preservation solution for 2 hours at 4°C. The donor aorta and pulmonary artery were anastomosed, end to side, to the recipient's abdominal aorta and inferior vena cava, respectively. During the transplantation procedure, the duration of warm ischemia was maintained constant. At 60 days, the abdomen was opened, and the allografts were harvested.

Histomorphometric Quantification of CAV Area and Parenchymal Rejection

The severity of CAV and parenchymal rejection was calculated by an independent observer who was blinded to the treatment protocol. Hearts were fixed in 10% formalin, paraffin-embedded, and sectioned transversely at the maximal circumference of the ventricle. Sections were cut (5 μm) and stained with elastica van Gieson highlighting the internal elastic lamina (IEL). Arteries that had a well-defined smooth muscle cell layer and IEL in the vascular wall were traced. Every complete cross section of the traceable arteries was calculated. Images of elastin-stained sections were captured with a Sony DXC-970 MD 3CCD color camera affixed atop a Zeiss imaging microscope. Images were captured by using this hardware and processed by using a Zeiss image-analysis program. Planimetered areas were calculated by image-analysis software. The percentage of luminal obliteration was determined in vessels that did not appear artifactually distorted by compressing or sectioning artifacts. The area encompassed by the lumen and IEL was traced, and the area of luminal stenosis in each section was calculated according to the following formula: luminal occlusion=(IEL area−luminal area)/IEL area. Grafts were also evaluated by standard hematoxylin and eosin stains to assess parenchymal rejection, which was graded by using a myocardial histological rejection scale (0, no mononuclear cell infiltration; 1, faint and limited mononuclear cell infiltration; 2, moderate mononuclear cell infiltration; and 3, severe and diffuse mononuclear cell infiltration).[14,18] Scores are reported as either the mean rejection score or the mean±SEM percent luminal occlusion score.

Northern Blotting

After tissue homogenization, total RNA (12 μg per lane) was subjected to electrophoresis in 0.8% agarose-formaldehyde gels and transferred to Duralon-UV membranes (Stratagene). Membranes hybridized with a ^{32}P-labeled cDNA probe for Egr-1 or Sp-1[19] were subsequently exposed to Kodak Biomax film (Eastman Kodak) at −80°C. Membranes were then stripped and rehybridized with radiolabeled human β-actin cDNA as a control for RNA loading.

Western Blotting

After tissue homogenization in the presence of a protease inhibitor cocktail tablet (Roche), proteins (20 μg) were loaded into each lane of an SDS-polyacrylamide gel. The gel was then electrophoresed, and proteins were transferred electrophoretically to nitrocellulose membranes. Immunoblotting was performed by using primary rabbit anti-mouse Egr-1 IgG (Santa Cruz) or rabbit anti-mouse Sp-1 IgG (Santa Cruz) antibodies. Secondary detection of primary antibody localization was accomplished by using a horseradish peroxidase–conjugated goat anti-rabbit whole IgG (Sigma Chemical Co). Final detection of immunoreactive bands was performed by using the enhanced chemiluminescence Western blotting system (Amersham International).

Immunohistochemistry

Sections were first stained with primary antibodies, a rabbit anti-mouse Egr-1 IgG (1:200 dilution, Santa Cruz), a rabbit anti-mouse PDGF-A IgG (1:100 dilution, Santa Cruz), a hamster anti-mouse ICAM-1 IgG (1:50 dilution, PharMingen), or a rat anti-mouse vascular cell adhesion molecule-1 (VCAM-1) IgG (1:100 dilution, PharMingen). Sites of primary antibody binding were visualized with biotinylated goat anti-rabbit IgG (1:100 dilution, Sigma), biotinylated mouse anti-hamster IgG (1:50 dilution, PharMingen), or biotinylated rabbit anti-rat IgG (1:100

dilution, Sigma), and ExtraAvidin peroxidase conjugate (1:100 dilution, Sigma) and developed in 3,3'-diaminobenzidine (Vector Laboratories). Nuclei were counterstained with hematoxylin.

Donor-Reactive Alloantibodies

Measurement of donor-reactive alloantibodies was performed as described previously.[20] Briefly, recipient serum was assayed for the presence of donor-reactive IgG alloantibodies by incubating serum (diluted 1:20 with buffer) in the presence of target donor splenocytes. This was followed by incubation with fluorescein isothiocyanate (FITC)-conjugated, $F(ab')_2$, Fc fragment–specific, goat anti-mouse IgG (Jax Laboratories) and phycoerythrin-conjugated anti-mouse CD3 monoclonal antibody (PharMingen). Flow cytometry data were collected by using a FACScan flow cytometer (Becton Dickinson), and only viable cells were analyzed. A 2-parameter display of FITC anti-IgG versus phycoerythrin anti-CD3 was generated, and data were analyzed by using CellQuest software. Serum from B10A mice sensitized by subcutaneous injection of C57BL/6J splenocytes was used as a positive control.

Ex Vivo Delivery of FITC-Labeled ODNs

FITC-labeled phosphorothioate antisense ODNs were synthesized by Operon Technologies. Flushing and preservation with use of the FITC-labeled ODNs was performed at the same concentration, volume, and vehicle as described for the other ODN experiments. Grafts were harvested at day 7 after delivery of the FITC-labeled ODNs and transplantation. Sections were cut (6 μm) and examined by fluorescence microscopy.

Statistical Analysis

All statistical comparisons were performed by using commercially available statistical software (STAT VIEW-J 5.0, SAS Institute) on a Macintosh PowerPC computer. One-way ANOVA was used to compare different conditions among the groups. Values are expressed as mean±SEM, with differences considered statistically significant at $P<0.05$.

Results

Expression of Egr-1 in Cardiac Allografts or Isografts

Egr-1 mRNA levels and mRNA levels of another zinc finger family transcription factor (Sp-1) were examined 60 days after transplantation (Figure 1A). These blots revealed high levels of Egr-1 mRNA in allografts compared with low levels of Egr-1 mRNA in isografts. In contrast to the observed induction of Egr-1 in allografts, there was no increase in Sp-1 either in allografts or in isografts. Immunohistochemistry revealed that Egr-1 antigen and one of its downstream gene targets (PDGF-A) were markedly increased in allograft coronary artery endothelial and smooth muscle cells, although they were virtually undetectable in naive hearts or isografts (Figures 1B through 1G).

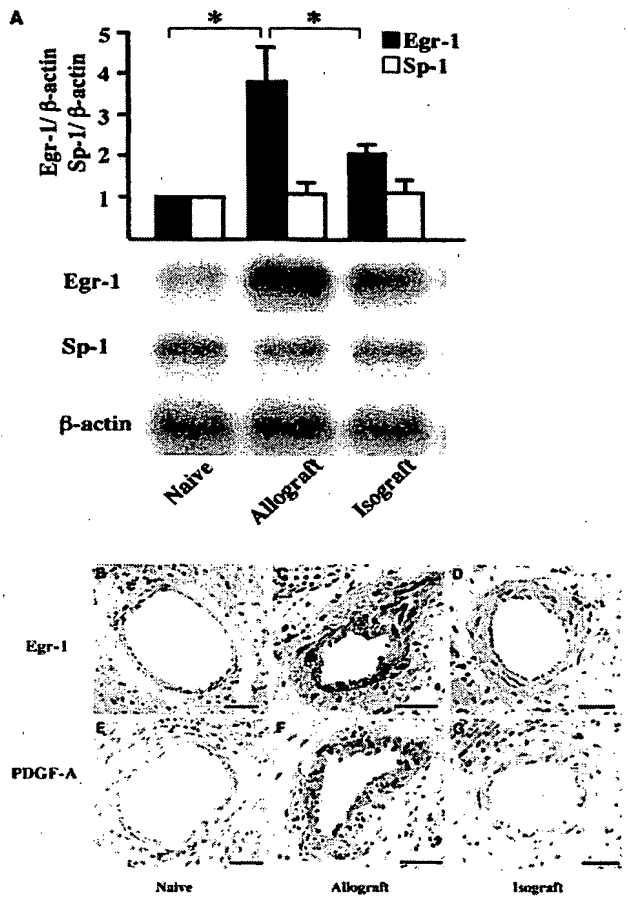

Figure 1. Egr-1 expression in cardiac allografts or isografts. Samples from allografts or isografts were collected 60 days after heterotopic transplantation of hearts obtained from donor C57BL/6J or B10A mice transplanted into recipient B10A mice, respectively. Northern analysis was performed with ^{32}P-labeled probes for Egr-1, Sp-1, and β-actin, as shown in panel A. A representative blot is shown. Quantitative densitometric data (expressed as Egr-1/β-actin or Sp-1/β-actin mRNA levels relative to naive heart) from multiple experiments (n=4) are shown as mean±SEM. *$P<0.05$. Representative vessels from naive hearts, isografts, or allografts stained with a primary antibody directed against Egr-1 are shown in panels B through D; PDGF-A immunoreactivity is shown in panels E through G. Positive immunoreactivity is brown (diaminobenzidine). Bar=50 μm.

Effect of Graft Egr-1 Expression on Late Development of CAV and Parenchymal Rejection

Elastin-stained sections were used to evaluate the frequency and severity of atherosclerotic lesion development. Although most vessels in allografts from both wild-type and Egr-1–deficient donors exhibited some degree of intimal thickening, the severity of lesions was significantly decreased (4.6-fold reduction) in allografts from Egr-$1^{-/-}$ donors compared with allografts from Egr-$1^{+/+}$ controls (Figures 2A through 2C). To highlight the severity of cell infiltration in myocardium, allograft sections were stained with hematoxylin and eosin. Severe cell infiltration was observed in Egr-$1^{+/+}$ allografts (Figure 2D). However, Egr-$1^{-/-}$ allografts showed markedly attenuated mononuclear cell infiltration, as scored by a blinded observer on the basis of rejection criteria[14,18] (Figure 2E). Rejection scores were statistically less in Egr-$1^{-/-}$ allografts than in Egr-$1^{+/+}$ allografts (Figure 2F). These data strongly support a participatory role for Egr-1 in cardiac allograft parenchymal rejection as well as in the development of CAV.

Egr-1 Regulation of Adhesion Molecules and Growth Factors

ICAM-1 and VCAM-1 are likely to be key mediators of atherosclerosis because their expression is inducible in ath-

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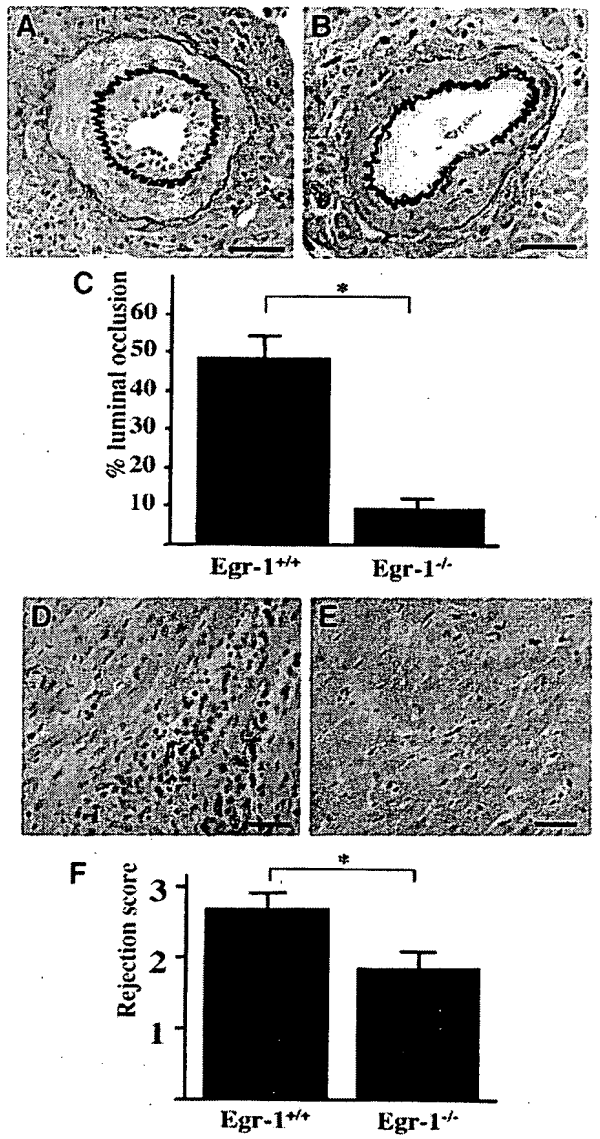

Figure 2. Effect of graft Egr-1 expression on late development of CAV and parenchymal rejection. Representative vessels from allografts were harvested 60 days after heterotopic transplantation and stained with elastica van Gieson obtained from donor mice of the indicated genotype (Egr-$1^{+/+}$ [A] or Egr-$1^{-/-}$ [B]) transplanted into the abdomen of a recipient mouse. Degree of CAV was objectively quantified histomorphometrically by using a computer-based imaging system (C). Representative myocardium was stained with hematoxylin-eosin from donor allografts of the indicated genotype (Egr-$1^{+/+}$ [D] or Egr-$1^{-/-}$ [E]). Parenchymal rejection (F) was graded by using a myocardial histological rejection scale (from 0 [no rejection] to 3 [severe rejection]). Data are expressed as mean±SEM. Numbers of transplants were 8 each. Bar=50 μm. *$P<0.05$.

erosclerotic lesions and because they have a high avidity for activated leukocytes. Of various candidate growth factors, PDGF has been studied as another key mediator of atherogenesis because it elicits a strong mitogenic response in smooth muscle cells and localizes quite strongly to atherosclerotic lesions. Given that Egr-1 has been implicated in the

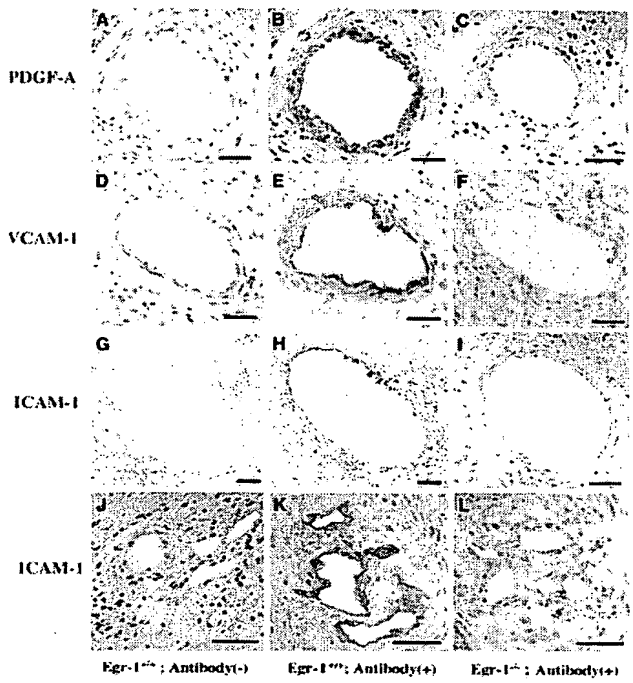

Figure 3. Effect of graft Egr-1 genotype on expression of PDGF-A, VCAM-1, and ICAM-1 antigen. Shown are representative vessels from allografts harvested 60 days after heterotopic transplantation of hearts obtained from donor mice transplanted into the abdomen of recipient B10A mice, stained without (A, D, G, and J) or with (B, C, E, F, H, I, K, and L) a primary antibody directed against the epitope labeled along the ordinate. The Egr-1 genotype of the donor mouse is listed along the abscissa. All recipients were wild type. Positive immunoreactivity is brown (diaminobenzidine). Bar=50 μm.

induced expression of adhesion molecules and growth factors, we hypothesized that the diminished induction of these proatherogenic mediators in Egr-1–null mice might account for the reduced CAV lesional development that was observed after cardiac transplantation. To test this hypothesis, cardiac allotransplants were performed by using Egr-1–competent or –null hearts and then examining them at 60 days for the expression of ICAM-1, VCAM-1, and PDGF-A. PDGF-A protein expression was found on both endothelial cells and smooth muscle cells of coronary arteries (Figure 3B). Hearts of Egr-$1^{+/+}$ mice displayed prominent immunoreactivity for ICAM-1 and VCAM-1 on coronary artery endothelial cells. Of particular interest was the expression of VCAM-1 on coronary artery smooth muscle cells, which did not demonstrate ICAM-1 expression (Figures 3E and 3H). No immunostaining was seen in control sections stained in the absence of primary antibody (Figures 3A, 3D, and 3G). Expression of ICAM-1, VCAM-1, and PDGF-A was greatly diminished (in fact, nearly undetectable) in Egr-$1^{-/-}$ hearts compared with Egr-$1^{+/+}$ cardiac allografts (Figures 3C, 3F, and 3I). Because the site of entry of leukocytes into allograft tissue is postcapillary vessels, we also investigated the expression of ICAM-1 at these sites. ICAM-1 antigen expression was found to be significantly increased on postcapillary vessels in Egr-$1^{+/+}$ allografts (but not Egr-$1^{-/-}$ allografts) at 60 days (Figures 3J

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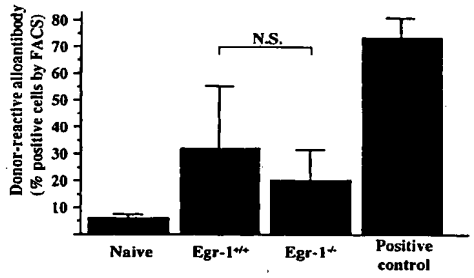

Figure 4. Donor-reactive alloantibodies in murine cardiac allograft recipients. To detect donor-reactive alloantibodies in sera from B10A recipient mice 60 days after heterotopic transplantation with either Egr-$1^{+/+}$ (n=8) or Egr-$1^{-/-}$ (n=8) donor hearts, B10A spleen cells were incubated with recipient serum and labeled with FITC-conjugated anti-mouse IgG, and cells falling within the T-cell gate ($CD3^+$) were analyzed by flow cytometry. Naive B10A mice (n=4) and B10A mice presensitized by subcutaneous injection of C57BL/6J splenocytes (n=4) were used as controls. N.S. indicates not significant.

through 3L). These findings are concordant with the hypothesis that induction of Egr-1 represents an upstream event in the setting of cardiac allograft transplantation, which triggers increased transcription of ICAM-1, VCAM-1, and PDGF-A, resulting in accelerated development of CAV.

Effect of Graft Egr-1 Expression on Donor-Reactive Alloantibodies

Donor-reactive alloantibodies, indicative of humoral allosensitization, have been shown by other groups to promote the development of occlusive vascular remodeling.[21] Therefore, we investigated the impact of Egr-1 on humoral immunity. Serum was obtained from recipients at day 60 after transplant and was tested for the presence of donor-reactive alloantibodies, as measured by their ability to bind donor splenocytes in flow cytometry studies. Sera from untreated naive mice and pooled sera from mice previously sensitized by injection of allogeneic splenocytes served as negative and positive controls, respectively. Mice transplanted with Egr-$1^{-/-}$ allografts developed low levels of donor-reactive alloantibodies, whereas mice transplanted with Egr-$1^{+/+}$ allografts developed moderate levels of donor-reactive alloantibodies (Figure 4). It is interesting that although there was no statistically significant difference between the development of alloantibodies whether the donor animal was Egr-$1^{-/-}$ or Egr-$1^{+/+}$, there did appear to be a trend toward reduced alloantibody formation in the Egr-1–null mice. These data suggest that humoral sensitization (as manifested by alloantibody production) is not likely to be the dominant reason for the clear-cut involvement of Egr-1 in the development of CAV, although a minor contributory role cannot be ruled out.

Localization of FITC-Labeled Phosphorothioate ODNs

By using methods identical to those used for the delivery of sense/antisense compounds, FITC-labeled phosphorothioate ODNs were delivered into allografts at the time of cardiac harvest/preservation to provide topographic localization of sites of incorporation. The delivery of FITC-labeled ODNs resulted in widespread distribution of fluorescence in vascular cells of allografts harvested at 7 days after transplantation (Figure 5).

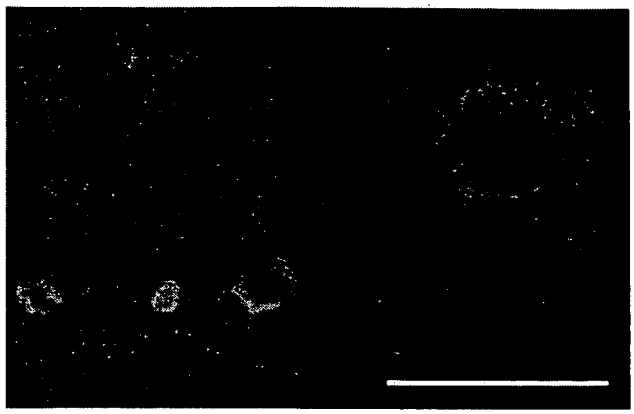

Figure 5. Representative fluorescence microscopic result of ex vivo delivery of FITC-labeled ODNs given at the time of donor heart preservation. A widespread distribution of fluorescence was noted in vascular cells of murine allografts harvested 7 days after transplantation. Bar=50 μm.

Effects of Antisense Egr-1 ODNs on Graft Egr-1 Expression

The next set of experiments was designed to test whether an antisense Egr-1 ODN delivered in the preservation fluid at the time of cardiac harvest could inhibit the expression of Egr-1 in cardiac allografts after transplantation. Samples were collected from (1) untreated naive hearts (rapidly excised from anesthetized/untreated mice), (2) hearts treated with scrambled-sequence ODN plus cationic liposomal carrier, or (3) hearts treated with antisense ODN plus cationic liposomal carrier. Egr-1 mRNA levels measured 60 days after transplantation were markedly elevated in the group of hearts preserved with scrambled-sequence ODNs. However, when hearts were preserved with antisense Egr-1 ODN but otherwise subjected to identical transplantation procedures, Egr-1 mRNA levels were diminished (Figure 6A). Concordant with these observations, analyses of the expression of Egr-1 protein showed that only antisense ODNs significantly blocked the increased level of Egr-1 protein, which was observed in the untreated or scrambled-sequence control groups after transplantation (Figure 6B). The effect of the antisense ODN to reduce Egr-1 was specific, in that a related transcription factor (Sp-1) was not affected.

To ascertain sites of Egr-1 expression 60 days after transplantation, immunohistochemical analysis was performed on transplanted cardiac allografts. When sections from the transplanted hearts preserved with scrambled-sequence ODNs were subjected to immunostaining procedures in the presence of the primary anti-mouse Egr-1 antibody, endothelial cells and smooth muscle cells in the neointima stained prominently (Figure 6D). In a negative control section, the primary antibody was omitted from the staining protocol, and immunoreactivity was not detected (Figure 6C). Antisense ODNs inhibited increased levels of Egr-1 immunoreactivity after transplantation (Figure 6E).

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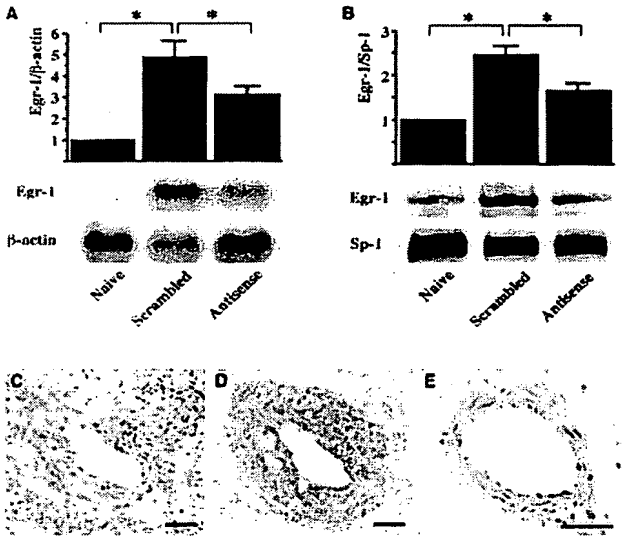

Figure 6. Inhibitory effects of antisense Egr-1 ODNs on graft Egr-1 expression. Samples were taken from naive hearts, from grafts treated with scrambled-sequence Egr-1 ODN (scrambled), or from grafts treated with antisense Egr-1 ODN (antisense). A, Effect of ODNs on graft Egr-1 mRNA levels, analyzed by Northern blotting. A representative blot is shown. Quantitative densitometric data (expressed as Egr-1/β-actin mRNA levels relative to naive heart) are shown as mean±SEM. *$P<0.05$ (n=4). B, Effect of ODNs on graft Egr-1 protein expression, analyzed by Western blotting. A representative blot is shown. Quantitative densitometric data (expressed as Egr-1/Sp-1 levels relative to naive heart) are shown as mean±SEM. *$P<0.05$ (n=6). C through E, Immunohistochemical localization of Egr-1 antigen expression. Representative sections of hearts treated with scrambled-sequence Egr-1 ODN stained without (C) or with (D) a primary anti-mouse Egr-1 antibody are shown. A representative allograft vessel after antisense Egr-1 ODN-supplemented preservation stained with a primary anti-Egr-1 antibody is shown (E). Positive immunoreactivity is brown (diaminobenzidine). Bar=50 μm.

These results demonstrate that the antisense Egr-1 ODN used in the present study effectively blocked the transplantation-associated induction of Egr-1 protein in cardiac allografts.

Effect of Antisense Egr-1 ODNs on Late Development of CAV and Parenchymal Rejection

Previous work in the heterotopic murine cardiac transplant model has shown that in the allogeneic combination B10A/C57BL/6J, characterized by major histocompatibility (class I and class II) mismatches, untreated allografts developed significant neointimal thickening (≈60% luminal obliteration) at the 60-day observation point.[3] Although severe neointimal thickening was observed in the coronary arteries of allografts treated with scrambled-sequence Egr-1 ODN (55.7±6.6% of the luminal obliteration), there was substantially less neointimal formation in allografts treated with antisense Egr-1 ODNs (37.1±4.3% of the luminal obliteration) (Figures 7A through 7C). This difference was observed despite identical immunosuppression, identical surgical procedures, identical preservation and ischemic times, and blinded administration of the two treatment regimens. These data indicate that Egr-1 blockade at the time of donor heart

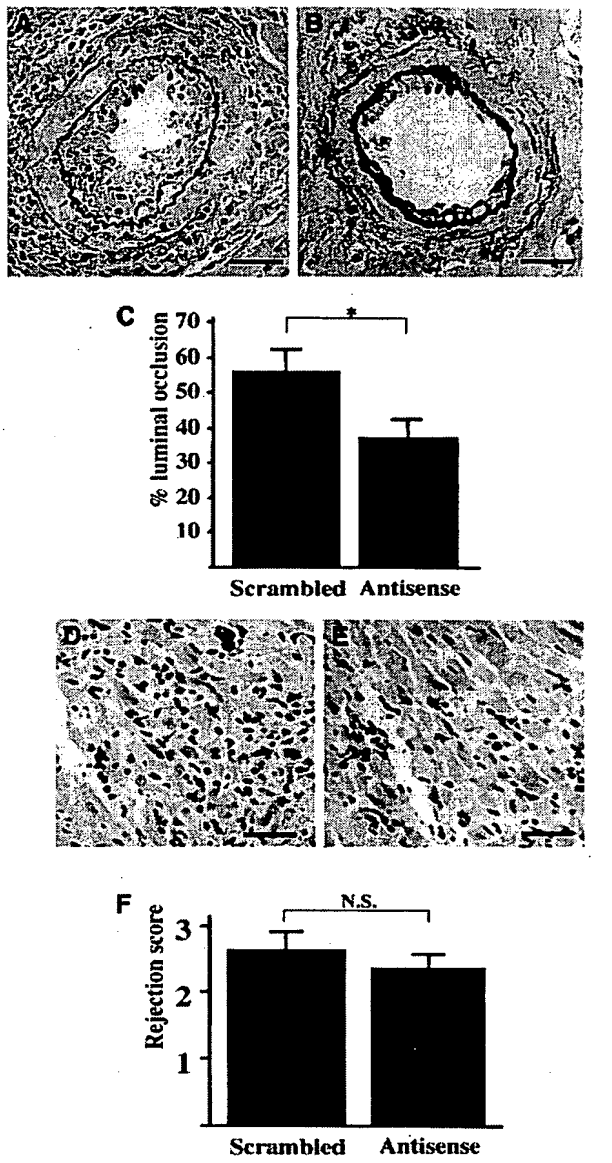

Figure 7. Effect of antisense Egr-1 ODNs on CAV. Samples were obtained 60 days after transplantation of B10A donor hearts into C57BL/6J recipients. Representative vessels from donor hearts were treated at the time of donor heart harvest with scrambled-sequence Egr-1 ODN and cationic liposomal carrier (A, scrambled) or antisense Egr-1 ODN and cationic liposomal carrier (B, antisense). The degree of CAV was objectively quantified histomorphometrically by using a computer-based imaging system (C). Representative hematoxylin-eosin–stained myocardial sections were from donor allografts that had been treated with scrambled-sequence Egr-1 ODN (D, scrambled) or antisense Egr-1 ODN (E, antisense). Parenchymal rejection (F) was graded by using a myocardial histological rejection scale (from 0 [no rejection] to 3 [severe rejection]). Data are expressed as mean±SEM. Numbers of transplants were as follows: scrambled, 7; antisense, 8. Bar=50 μm. *$P<0.05$.

preservation has a salutary effect on the graft vasculature. The severity of cell infiltration in myocardium was minimally (not significantly) less in antisense-treated allografts than in scrambled-sequence–treated allografts (Figures 7D through

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7F). These data suggest that the inhibitory impact of the antisense Egr-1 treatment strategy is greatest on the development of allograft vasculopathy, with less of an effect on parenchymal rejection.

Discussion

The development of chronic vascular rejection is patently multifactorial, with a prominent immunological component. It is also becoming more apparent that vascular injury secondary to ischemia/reperfusion injury can act as a trigger or an accelerant for the development of CAV.[5] In a study of >25 000 patients,[1] the long-term survival of human cardiac allografts was shown to be diminished when the donor organ was subjected to prolonged preservation. In fact, there was a linear relation between donor ischemia time and 1- and 5-year survival. More recently, kidneys subjected to prolonged travel time in transit between donor and recipient experienced more rejection than those that traveled shorter distances.[22] In a rat cardiac allograft model, early ischemic injury has been shown to accelerate the development of CAV and to increase the ultimate degree of luminal compromise.[4] Murine cardiac isografts developed a minor vasculopathy when donor hearts were subjected to ischemic injury at the time of transplantation, but CAV was greatly increased when heart transplantation was performed across an alloimmune barrier.[3] An implication of these data is that improving the early preservation/ischemia or reperfusion milieu might reduce the incidence or severity of CAV after heart transplantation. Because Egr-1 is a key early-activated transcription factor that ignites inflammatory and thrombotic cascades in ischemic vessels,[8,13] we hypothesized that CAV could be prevented by Egr-1 blockade. Genetic absence of this proximally acting transcription factor in lung vessels results in diminished expression of cytokines (such as MIP2, JE/MCP-1, and interleukin-1β), adhesion receptors (such as ICAM-1), and prothrombotic genes (such as tissue factor and plasminogen activator inhibitor-1) after an ischemic insult. Many of these are the very same pathways implicated in leukocyte trafficking and chronic rejection, leading us to determine whether activation/induction of Egr-1 might participate in the pathogenic development of CAV.

The present study shows for the first time that CAV can, at least in part, be triggered by Egr-1 induction and that this critical event can be diminished by a simple strategy of adding an antisense Egr-1 construct to the preservation fluid in which the donated heart is steeped. In the present study, the genetic or induced absence of the Egr-1 gene product not only reduces the severity of acute and chronic rejection but also reduces the coincident induction of ICAM-1, VCAM-1, and PDGF-A seen in control (Egr-1–expressing) grafts. These data are consonant with the data for human and rat cardiac allografts, in which transplantation-associated increases in glycoprotein adhesion receptor expression have been linked with rejection (with marked elevations in circulating levels of ICAM-1, VCAM-1, and P-selectin).[7] In addition, expression of growth factors such as PDGF-A is significantly increased in human cardiac allografts.[23] Because administration of monoclonal antibodies against adhesion molecules has been reported to prolong graft survival or induce tolerance,[24] it is reasonable to expect that Egr-1 may promote rejection by inducing a coordinated induction of genes producing products that recruit leukocytes as well as those that permit their retention at sites of rejection. Despite the virtual obliteration of CAV in the absence of Egr-1 expression in these experiments, it must be noted that observations were made only until the 60-day posttransplantation time point. It is possible that the time to vasculopathy may just be shifted by the absence of Egr-1, the absence of which might impose a delay but not a permanent obliteration of vasculopathy development.

The data shown in Figure 4 suggest the possibility (but do not prove) that mice lacking the Egr-1 gene mount a diminished humoral response to a transplanted cardiac allograft. The role of Egr-1 expression in the development of humoral alloimmunity is important to consider, because humoral alloimmunity is likely to be a major factor in the development of chronic vascular rejection. In studies performed in a mouse heart transplant model similar to those performed here, transplants between strains that produced alloantibodies developed coronary neointimal lesions that were more extensive than when transplants were performed between strains that did not exhibit this brisk humoral response.[21] In fact, treatment with donor antiserum in this model actually increased the CAV lesions.[21] There is also a defined role for cell-mediated immunity in the coronary endotheliitis that develops after cardiac allotransplantation, inasmuch as cardiac allografts transplanted into B-cell–deficient recipients demonstrated destruction of arterial media and adhesion of T cells and mononuclear cells to the endothelium, although the subsequent neointimal proliferative response was not particularly brisk.[25] When these data are considered in light of our own experiments, they suggest that that Egr-1 may contribute to the development of cellular and humoral alloimmunity.

There are several strategies that may be effective in inhibiting the pathological consequences of Egr-1 induction. DNA enzymes that specifically bind to and degrade Egr-1 are effective at preventing vascular smooth muscle proliferation and regrowth after mechanical vascular injury.[11] Because of potential concerns regarding specificity of the phosphorothioate ODN for the chosen target, scrambled-sequence phosphorothioate ODNs that have sizes and net charges identical to those of their antisense counterparts but do not form heteroduplexes with target mRNA were used for control conditions. In the present study, only antisense Egr-1 ODN but not scrambled-sequence Egr-1 ODN reduced both Egr-1 mRNA and protein levels, pointing to a direct inhibitory effect of this ODN on the targeted sequence, Egr-1. These data also reveal an interesting feature of the particular antisense approach taken: providing the antisense ODN solely at the time of organ preservation results in diminished expression of the Egr-1 target gene up to 2 months later. In a similar heart transplant model in mice, delivery of an E2F decoy at the time of graft preservation suppressed the expression of E2F and its associated cell-cycle–regulatory genes for up to 8 weeks of observation.[26] Although, presumably, the persistence of the antisense construct is abbreviated, it is likely that early reductions in graft vascular injury can be propagated over time, because diminished early inflammation is likely to

be somewhat self-perpetuating. Regardless of the persistence or nonpersistence of the antisense construct, these data clearly show a persistence of effect, on the target gene itself and its own downstream targets. These data are the first to identify Egr-1 induction as a potential unifying mechanism driving the ischemia-related acceleration of CAV development in cardiac allografts.

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References

1. Hosenpud JD, Bennett LE, Keck BM, Boucek MM, Novick RJ. The Registry of the International Society for Heart and Lung Transplantation: Eighteenth Official Report–2001. *J Heart Lung Transplant.* 2001;20: 805–815.
2. Hosenpud JD. Immune mechanisms of cardiac allograft vasculopathy: an update. *Transpl Immunol.* 1993;1:237–249.
3. Wang CY, Aronson I, Takuma S, Homma S, Naka Y, Alshafie T, Brovkovych V, Malinski T, Oz MC, Pinsky DJ. cAMP pulse during preservation inhibits the late development of cardiac isograft and allograft vasculopathy. *Circ Res.* 2000;86:982–988.
4. Knight RJ, Dikman S, Liu H, Martinelli GP. Cold ischemic injury accelerates the progression to chronic rejection in a rat cardiac allograft model. *Transplantation.* 1997;64:1102–1107.
5. Hancock WW, Buelow R, Sayegh MH, Turka LA. Antibody-induced transplant arteriosclerosis is prevented by graft expression of anti-oxidant and anti-apoptotic genes. *Nat Med.* 1998;4:1392–1396.
6. Holschermann H, Bohle RM, Zeller H, Schmidt H, Stahl U, Fink L, Grimm H, Tillmanns H, Haberbosch W. In situ detection of tissue factor within the coronary intima in rat cardiac allograft vasculopathy. *Am J Pathol.* 1999;154:211–220.
7. Koskinen PK, Lemstrom KB. Adhesion molecule P-selectin and vascular cell adhesion molecule-1 in enhanced heart allograft arteriosclerosis in the rat. *Circulation.* 1997;95:191–196.
8. Yan SF, Fujita T, Lu J, Okada K, Shan Zou Y, Mackman N, Pinsky DJ, Stern DM. Egr-1, a master switch coordinating upregulation of divergent gene families underlying ischemic stress. *Nat Med.* 2000;6:1355–1361.
9. Khachigian LM, Lindner V, Williams AJ, Collins T. Egr-1-induced endothelial gene expression: a common theme in vascular injury. *Science.* 1996;271:1427–1431.
10. McCaffrey TA, Fu C, Du B, Eksinar S, Kent KC, Bush H Jr, Kreiger K, Rosengart T, Cybulsky MI, Silverman ES, Collins T. High-level expression of Egr-1 and Egr-1-inducible genes in mouse and human atherosclerosis. *J Clin Invest.* 2000;105:653–662.
11. Santiago FS, Lowe HC, Kavurma MM, Chesterman CN, Baker A, Atkins DG, Khachigian LM. New DNA enzyme targeting Egr-1 mRNA inhibits vascular smooth muscle proliferation and regrowth after injury. *Nat Med.* 1999;5:1264–1269.
12. Wada Y, Suzuki J, Kawauchi M, Kurabayashi M, Tsukioka K, Zhang T, Endoh M, Takayama K, Nagai R, Takamoto S, Isobe M, Amano J. Early growth-response factor 1 and basic transcriptional element-binding protein 2 expression in cardiac allografts. *J Heart Lung Transplant.* 2001;20:590–594.
13. Okada M, Fujita T, Sakaguchi T, Olson KE, Collins T, Stern DM, Yan SF, Pinsky DJ. Extinguishing Egr-1-dependent inflammatory and thrombotic cascades following lung transplantation. *FASEB J.* 2001;15: 2757–2759.
14. Suzuki J, Isobe M, Morishita R, Aoki M, Horie S, Okubo Y, Kaneda Y, Sawa Y, Matsuda H, Ogihara T, Sekiguchi M. Prevention of graft coronary arteriosclerosis by antisense cdk2 kinase oligonucleotide. *Nat Med.* 1997;3:900–903.
15. Toda K, Kayano K, Karimova A, Naka Y, Fujita T, Minamoto K, Wang CY, Pinsky DJ. Antisense intercellular adhesion molecule-1 (ICAM-1) oligodeoxyribonucleotide delivered during organ preservation inhibits posttransplant ICAM-1 expression and reduces primary lung isograft failure. *Circ Res.* 2000;86:166–174.
16. Muthukkumar S, Han SS, Rangnekar VM, Bondada S. Role of Egr-1 gene expression in B cell receptor-induced apoptosis in an immature B cell lymphoma. *J Biol Chem.* 1997;272:27987–27993.
17. Wang CY, Naka Y, Liao H, Oz MC, Springer TA, Gutierrez-Ramos JC, Pinsky DJ. Cardiac graft intercellular adhesion molecule-1 (ICAM-1) and interleukin-1 expression mediate primary isograft failure and induction of ICAM-1 in organs remote from the site of transplantation. *Circ Res.* 1998;82:762–772.
18. Billingham ME, Cary NR, Hammond ME, Kemnitz J, Marboe C, McCallister HA, Snovar DC, Winters GL, Zerbe A. A working formulation for the standardization of nomenclature in the diagnosis of heart and lung rejection: Heart Rejection Study Group. *J Heart Lung Transplant.* 1990;9:587–593.
19. Yan SF, Lu J, Zou YS, Soh-Won J, Cohen DM, Buttrick PM, Cooper DR, Steinberg SF, Mackman N, Pinsky DJ, Stern DM. Hypoxia-associated induction of early growth response-1 gene expression. *J Biol Chem.* 1999;274:15030–15040.
20. Pelletier RP, Adams PW, Hennessy PK, Orosz CG. Comparison of crossmatch results obtained by ELISA, flow cytometry, and conventional methodologies. *Hum Immunol.* 1999;60:855–861.
21. Russell PS, Chase CM, Winn HJ, Colvin RB. Coronary atherosclerosis in transplanted mouse hearts, II: importance of humoral immunity. *J Immunol.* 1994;152:5135–5141.
22. Mange KC, Cherikh WS, Maghirang J, Bloom RD. A comparison of the survival of shipped and locally transplanted cadaveric renal allografts. *N Engl J Med.* 2001;345:1237–1242.
23. Zhao XM, Yeoh TK, Frist WH, Porterfield DL, Miller GG. Induction of acidic fibroblast growth factor and full-length platelet-derived growth factor expression in human cardiac allografts: analysis by PCR, in situ hybridization, and immunohistochemistry. *Circulation.* 1994;90: 677–685.
24. Isobe M, Yagita H, Okumura K, Ihara A. Specific acceptance of cardiac allograft after treatment with antibodies to ICAM-1 and LFA-1. *Science.* 1992;255:1125–1117.
25. Russell PS, Chase CM, Colvin RB. Alloantibody- and T cell-mediated immunity in the pathogenesis of transplant arteriosclerosis: lack of progression to sclerotic lesions in B cell-deficient mice. *Transplantation.* 1997;64:1531–1536.
26. Kawauchi M, Suzuki J, Morishita R, Wada Y, Izawa A, Tomita N, Amano J, Kaneda Y, Ogihara T, Takamoto S, Isobe M. Gene therapy for attenuating cardiac allograft arteriopathy using ex vivo E2F decoy transfection by HVJ-AVE-liposome method in mice and nonhuman primates. *Circ Res.* 2000;87:1063–1068.